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A MULTIPLEX REVERSE TRANSCRIPTASE PCR ASSAY FOR DETECTION OF RESPIRATORY VIRUSES IN A TERTIARY CARE CENTER IN TRIPURA

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ABSTRACT Acute respiratory illnesses (ARIs) are a leading cause of infectious disease-related morbidity, hospitalization, and mortality among children and adults worldwide, particularly in developing countries. The purpose of this study is to detect common viral agent causing acute respiratory tract illness in children and adult population of Tripura, to determine the proportion of Influenza, Respiratory Syncytial Virus (RSV) and other viruses causing acute respiratory tract illness using Multiplex reverse transcriptase PCR. A two year cross sectional study from August 2016 to July 2018 was performed in patients attending OPD and IPD of this tertiary care centre with symptoms like acute fever 38°C or more, with any one of respiratory symptoms (such as sore throat, cough, Rhinorrhoea and breathlessness). Naso-pharyngeal swab was collected from patients below one year of age, and oral pharyngeal swabs/aspirate were collected from adult and paediatric population (1year to 70 years). Multiplex RT-PCR method was performed to detect the viral agents. A total of 289 patients were enrolled in this study from August 2016 to July 2018. Among the population 65.06% (n=188) was children and 34.94% (n=101) adult. The number of viral pathogens detected was 24% (69/289) patients. In children the most frequently detected viral agent was Influenza B (65.22%), in the adult group H1N1 predominated with 60.87%. Multiplex one step RT-PCR shows greater sensitivity and is useful for quickly differentiating between influenza types and subtypes. This will help in ongoing surveillance programme for influenza like illness and determining public health measures.

KEYWORDS : Acute respiratory illnesses, Multiplex one step RT-PCR, Respiratory syncytial Virus (RSV), Influenza A and B viruses.

INTRODUCTION:

Acute respiratory illnesses (ARIs) are a leading cause of infectious disease-related morbidity, hospitalization, and mortality among children and adults worldwide, particularly in developing countries[1]. Regardless of geographic location, the most common etiologic agents of ARIs are viruses [2]. The most frequently implicated viruses among hospitalized children are Respiratory Syncytial Virus (RSV), Human metapneumovirus (hMPV), Influenza A and B viruses (InfV A and InfV B), Parainfluenza viruses (PIV1-2-3) and Adenoviruses (AdV) [3,4,5,6]. Respiratory infections caused by above mentioned viruses usually present with clinical features that are nearly indistinguishable [7]. The difficulty also results from a lack of rapid diagnostic technique to differentiate specific viruses among ARIs [8]. Accurate estimates of the relative contribution of viral pathogens to ARI deaths and the factors modulating their activity are important for a timely and effective implementation of preventive interventions and optimal treatment routines, especially in light of recent efforts at vaccine development for other respiratory viruses [9,10].

The high burden of ARIs is disproportionate in developing countries, where it is estimated that over 1.5 million people die from acute respiratory infections globally, every year[11,12].

In India, a study conducted by Agrawal et al., published in 2009[13], was one of the first reports of a systematic surveillance of respiratory viruses with seasonal correlation and prevalence rates from Eastern India. Overall, Influenza A (IAV), Influenza B (IBV) and respiratory syncytial virus (RSV) were detected in 121 (11.09 %), 59 (5.41 %) and 95 (8.71 %) samples, respectively.

Viral Research Diagnostic Laboratory (VRDL) of this tertiary care centre perform standard molecular techniques for identification of circulating viruses among the patients suffering from ARTI. The standardisation of molecular methods such as Reverse transcriptase polymerase chain reaction (RT-PCR) has facilitated rapid and sensitive simultaneous diagnostic detection of the variety of viruses causing respiratory tract infection.

This is a 2-year cross-sectional study to detect viral aetiologies, clinical features and epidemiological patterns of ARIs in hospitalized as well as outdoor patients using established protocol of nucleic acid amplification techniques.

The purpose of this study is to detect common viral agent causing acute respiratory tract illness in children and adult population of Tripura, to determine the proportion of Influenza, Respiratory Syncytial Virus (RSV) and other viruses causing acute respiratory tract illness using Multiplex reverse transcriptase PCR and to determine the clinico-demographic profile of the detected viral agents among the study group. The study was undertaken as part of VRDL activity as per approval of Institutional Ethical Committee.

STUDY DESIGN:

A two year cross sectional study from August 2016 to July 2018 was performed. In this study, naso-pharyngeal swab was collected from patients below one year of age, and oral pharyngeal swabs/aspirate were collected from adult and paediatric population (1year to 70 years) of Tripura attending OPD and IPD of this tertiary care centre. Patients with symptoms like acute fever 38°C or more, with any one of respiratory symptoms (such as sore throat, cough, rhinorrhoea and breathlessness) are included in this study. The specimens were

transported immediately to the laboratory in sterile viral transport media (VTM) (Hi-media) then aliquoted and immediately frozen at -80°C until further processing.

METHODS

RNA extraction and Detection of respiratory viruses

RNA was extracted using a commercial reagent (QIAamp® Viral RNA mini kit, Qiagen), starting from clinical specimen volume of 140 µl and final elution volume of 60 µl. The extracts were tested immediately, if this was not possible, they were divided into aliquots and kept frozen at −80 ◦C. Each aliquot was used only once to avoid the loss of viral genomic material during repetitive freezing and thawing.

Multiplex one step RT-PCR:

Multiplex RT-PCR method was performed as per National Institute of Virology (NIV) protocol targeting 5 respiratory viruses: Influenza A (Pandemic H1N1 and Seasonal H3N2), Influenza B and RSV (A and B). The One-step RT-PCR kit from QIAGEN performed in this multiplex RT-PCR. This RT-PCR performed in two set, the reaction mixture for first set contained 5 µl of 5x RT-PCR buffer (2.5mM MgCl2), 1 µl 10 mM dNTPs mix, 1 µl of SET 1 10 mM forward and reverse primers mix and 1 µl of enzyme mix. A 10 µl aliquot of RNA extract was added to give a final volume of 25 µl. 1 µl of SET 2 10 mM forward and reverse primers mix using in second set reaction mixture and other components are used as same as reaction set 1.The cycling conditions for the RT-PCRs were: an initial cycle at 55 ◦C for 30 min and 94 ◦C for 10 min; followed by 35 cycles at 94 ◦C for 20 s, 55 ◦C for 1 min and 68 ◦C for 1 min; and a final incubation at 68 ◦C for 7 min. PCR products were visualized after electrophoresis on an ethidium–bromide stained 2% agarose gel. All statistical tests were conducted at Graph pad prism 7 during the study period.

SET 1 Primers

Virus	Primer	Oligonucleotide sequence 5'-3'	Gene	Amplicon size
Influenza A	Forward Primer	GAGGCTCTCATGGARTGG CTAIIIIIAAGACCAAT	M	450
	Reverse Primer	CATRGCCTTAGCYGTAGTG CTGGIIIIIACCATTCTG		
Influenza B	Forward Primer	GAGAAGGCAAAGCAGAA CTAGCAGIIIIITTACACTGT TG		515
	Reverse Primer	CATTGTTTTTGCTGTGTTC ATAGCTGIIIIIATCTGCATTT C		
Sesonal H3N2	Forward Primer	CATAGAAAATGGTTGGGA GGGAATGIIIIAYGGYTGGT AC	HA	303
	Reverse Primer	AGATCAATTGTATGTTGGT TYTCCAGIIIIACAAGAAGC TC		
Pandemic H1N1	Forward Primer	GGCCCAATCATGACTCGA ACAAAIIIITAACGGCAG		599
	Reverse Primer	CGGGATATTCCTTAATCCT GTRGCIIIICTCAATTTT		

SET 2 Primers

Virus	Primer ID	Oligonucleotide sequence 5'-3'	Gene	Amplicon size
Influenza B	Forward Primer	GAGAAGGCAAAGCAGAA CTAGCAGIIIIITTACACTGT TG	M	515
	Reverse Primer	CATTGTTTTTGCTGTGTTC ATAGCTGIIIIIATCTGCATTT C		
Influenza A	Forward Primer	GAGGCTCTCATGGARTGG CTAIIIIIAAGACCAAT		450
	Reverse Primer	CATRGCCTTAGCYGTAGTG CTGGIIIIIACCATTCTG		
RSV A	Forward Primer	GTGCTCTACTATCCACAAA CAAGGIIIIIGTCAGCTTA	F	248
	Reverse Primer	CATATAAGTGCTTACAGGT GTAGTTACIIIIICATTAACA C		
RSV B	Forward Primer	GCTGGAAATTACACACAT CACCTCIIIIIACCACCAAC		316
	Reverse Primer	CCATAGCATGACACTATAG CTCCIIIIIAAGTAATTACTG		

RESULTS

A total of 289 patients were enrolled in this study from August 2016 to July 2018. Among the population 65.06% (n=188) was children and 34.94% (n=101) adult (Figure: 01), where 36% (104/289) were from IPD and 64% (185/289) from OPD. In IPD cases 60% (n=62) belongs to adult and 40% (n=42) paediatric population, whereas in OPD cases 18% (n=33) adult and 82% (n=152) from paediatric population. In view of age, study subjects ranged from 1 month to 70 years. Clinically all positive cases had fever, along with Rhinorrhoea (90%), sore throat (87%), Breathlessness (20%) and cough (16%). In provisions of seasonality Influenza B, RSV A and B showed seasonal variation with peaks during June to August. The H_1N_1 peaks during March to May and for H_3N_2 it was end of the year i.e. August to October (Figure: 02). The number of viral pathogens detected was 24% (69/289) patients. The positivity among children and adult were 67% (n=46) and 33% (n=23) respectively. In children the most frequently detected viral agent was Influenza B (65.22%), followed by RSV B (15.22%), RSV A (6.52%), H_3N_2 (6.52%) and H_1N_1 (6.52%) (Figure: 02). In the adult group H_1N_1 predominated with 60.87 % followed by H_3N_2 (31.82%) and Influenza B (9.09%) (Figure: 03).

Influenza B (70%) was the most common virus detected between 1 to 10 years. In the age group <1 year, RSVB (22%) was predominated. The age group between 31 to 40 years most frequent viral agent was H_1N_1. (Figure: 04).

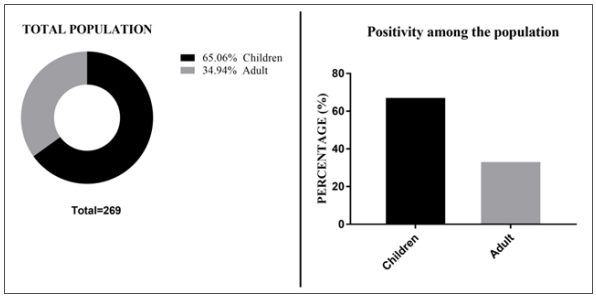

Figure: 01

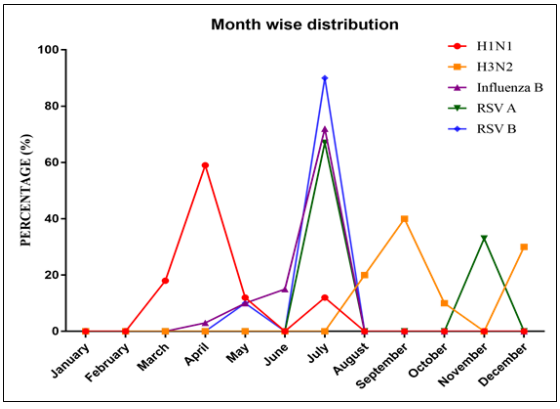

Figure: 02

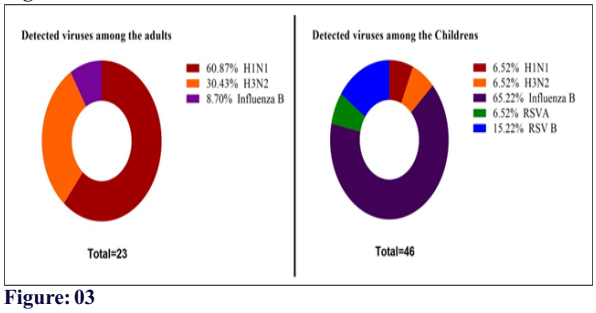

Figure: 03

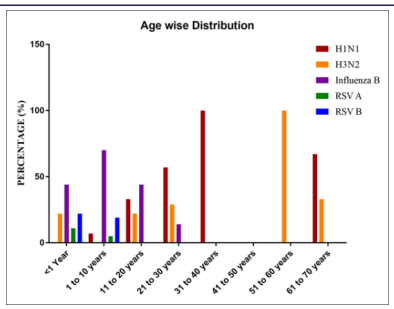

Figure: 04

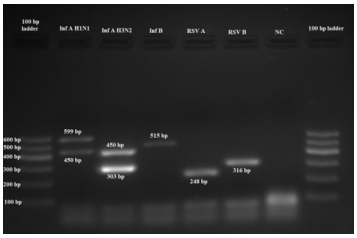

Figure: 05

Lane 1: 100 bp ladder (Qiagen).
Lane 2: Amplicon for Influenza A H1N1 showing band of 450 bp and 599 bp,
Lane 3: Influenza A H3N2 showing band of 450 bp and 303 bp.
Lane 4: Influenza B showing band of 515 bp.
Lane 5: RSV A showing band of 248 bp,
Lane 6: RSV B showing band of 316 bp
Lane 7: NC
Lane 8: 100 bp ladder (Qiagen).

DISCUSSION:

The rapid and accurate analysis of a wide range of viral agents is critical for aetiological investigations. The present study shows the viral etiologies among 24% of tested cases of ARIs in 269 hospitalized and outdoor patients in our setup enrolled during a period of 2 years (August 2016 to July 2018). This lower detection rate may be because of deficiency in proper sampling technique as it is a preliminary study and a wide scale study is necessary to establish proper frequency of the disease. However different study shows the actual viral aetiology as the viral load present in nasopharyngeal and throat swab may be less than nasopharyngeal aspirate [14]. Multiplex one step RT-PCR method was used in our study for detection of respiratory viruses. RT-PCR shows greater sensitivity than viral culture, may be used as a confirmatory test, and is useful for quickly differentiating between influenza types and subtypes [15].

In this study, multiple viral agents, including Influenza A (Pandemic H1N1 and seasonal H3N2) and Influenza B, RSV A and B, were analysed in a large population, with the goal of providing comprehensive data for viral infection in adults and children with ARTIs. Out of 269 total cases, 65.06% were children and 34.94% were adults, out of which, 68 patients (25.2%) were positive for viral infection. Among them 26.3% and 23.4% were positive in children and adults respectively.

In paediatric group, Influenza B was the most frequently detected virus, accounted for 65.22% of infections, RSV B is the second highest viral agent (15.22%) followed by H1N1, H3N2 and RSV A were found to be of equal positivity (6.52%). As observed in a study conducted in AIIMS[16], RSV was found to be most predominant agent in less than 1-year age group, but in our study Influenza B was the most predominant one.

In adult group, pandemic H1N1 was the most predominant virus, accounting 59.09% of infections followed by H3N2 (31.82%) and Influenza B (9.09%). In adult group between 31 to 40 years of age, H1N1 was found to be the only viral agent causing ARTIs which again become predominant in the age group of 61 to 70 years (67%), and in the age group between 51 to 60 years H3N2 is found to be the most predominant one.
Influenza virus was the most common agents detected throughout the year. The infection caused by Influenza virus usually occurs in the winter and most unlikely in summer in temperate zones [17,18]. In our study we have found that the prevalence rate of Influenza virus pandemic H1N1 was much higher during summer season i.e. from March to June and the number of positive cases of Influenza A seasonal H3N2 rise in August to September [19] (figure 4), the Influenza A seasonal H3N2 cases declined sharply during the first week of October. The positivity of Influenza B showed seasonal variation with peaks during June to August. RSV (A and B) infection peak was observed in the rainy season i.e. June to August as compared to a study done in Japan between 2012 to 2015, where the RSV activity is higher in July [20].

CONCLUSION:

This may not reflect the actual viral aetiology as the viral load present in nasopharyngeal and throat swab may be less as per other studies [20]. Influenza B, RSV A and RSV B showed seasonal variation with peaks during June to August. H1N1 peaks during March to May and H3N2 peaks during August to October. Active surveillance and proper management is required for this kind of study to restrict the use of antibiotics.

- This study for the first time elucidated the spectrum of viral agents causing ARI in Tripura.
- Among paediatric age group Influenza B is predominant when in adult most common is H1N1.
- Rapid and accurate diagnosis of respiratory viruses is utmost important to improve patient management and direct therapy following a specific diagnosis.
- Identification of viral agents will limit unnecessary antibiotic usage and prevent nosocomial spread of virus among high risk individuals.
- Molecular testing may be undertaken for surveillance to guide infection control practice or when high pathogenic emerging viral agents are suspected.

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